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Evidence that Synthesis of the *Saccharomyces cerevisiae* Mitochondrially Encoded Ribosomal Protein Var1p May Be Membrane Localized

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The 5′-untranslated leaders of mitochondrial mRNAs appear to localize translation within the organelle. *VAR1* is the only yeast mitochondrial gene encoding a major soluble protein. A chimeric mRNA bearing the *VAR1* untranslated regions and the coding sequence for pre-Cox2p appears to be translated at the inner membrane surface. We propose that translation of the ribosomal protein Var1p is also likely to occur in close proximity to the inner membrane.

The yeast mitochondrial genome codes for eight major polypeptides, seven of which are components of respiratory complexes located in the mitochondrial inner membrane (28). The eighth translation product, Var1p, is a soluble protein and a structural component of the small subunit of the mitochondrial ribosome, necessary for ribosome assembly (9, 12, 26, 27). Translation of many, if not all, mRNAs encoded by the yeast mitochondrial genome depends on the action of nuclear-encoded, inner membrane-bound, mRNA-specific translational activators (4, 7, 8, 13, 15, 17). These activators are thought to help target translation of mitochondrially encoded mRNAs to sites of respiratory complex assembly on the inner membrane through their interactions with mRNA 5′-untranslated leaders (UTLs) (4, 17, 21).

Little is known about the location of the *VAR1* mRNA translation or its activation. For example, is the *VAR1* mRNA translated at the surface of the inner membrane despite the fact that its product is assembled into the ribosome? Our laboratories have shown previously that synthesis of the membrane proteins Cox2p and Cox3p, subunits of the multimeric enzyme cytochrome *c* oxidase, can be directed by chimeric mRNAs bearing the untranslated regions (UTRs) of the *VAR1* mRNA (21). However, the vast majority of Cox2p and Cox3p synthesized under these conditions was rapidly degraded, leading to decreased steady-state levels of the proteins and decreased respiratory growth rates. Thus, the *VAR1* UTRs of the chimeric mRNAs apparently caused mislocalized synthesis of Cox2p and Cox3p, reducing the efficiency with which they are assembled into cytochrome *c* oxidase (21).

Cox2p is synthesized as a larger precursor, pre-Cox2p (23), whose N-terminal 15 amino acids are removed by the Imp proteolytic complex after translocation through the inner membrane to the intermembrane space (1, 18, 19, 22). Pulse-labeled Cox2p translated from the chimeric *var1*::*COX2* mRNA exhibited the same electrophoretic mobility as the wild-type protein (21). If pre-Cox2p encoded by the chimeric mRNA were synthesized in a soluble matrix milieu by free ribosomes, it might be subsequently clipped at the N or C terminus by nonspecific matrix proteases to yield a species with the same mobility as mature Cox2p. However, if pre-Cox2p encoded by the chimeric mRNA were translated at the inner membrane, then its N terminus could be rapidly translocated through the membrane and processed by Imp in the intermembrane space. To distinguish these possibilities, we asked whether the deletion of *IMP1*, which encodes a catalytic subunit of the Imp complex, would prevent processing of pre-Cox2p translated from the *var1*::*COX2* mRNA.

We constructed strains (Table 1), using standard methods (3, 5, 10), that contained a nuclear gene supplying Var1p on a plasmid (pAM2) (20), the *COX2* coding sequence inserted at the *VAR1* locus, and a replacement of the endogenous *COX2* coding sequence by the mitochondrial reporter gene $ARG8^m$ (24), encoding an arginine biosynthetic enzyme. Control strains had $ARG8^m$ in place of *VAR1* and the wild-type *COX2* gene. Mitochondrial translation was followed in vivo by pulse-labeling cells with [^{35}S]methionine for 10 min in the presence of cycloheximide as described previously (3), except that cells were grown initially in synthetic complete medium lacking uracil with 2% raffinose and then shifted to liquid 1% yeast extract–2% Bacto Peptone–2% raffinose for 5 h, and protease inhibitors (EDTA-free Complete; Roche) were added during cell disruptions.

Autoradiography of pulse-labeled proteins from diploid strains, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, confirmed that the *var1*::*COX2* mRNA was translated and that the labeled product had the same mobility as mature wild-type Cox2p (Fig. 1, lanes 1 and 2). Labeling of the *var1*::*COX2* mRNA translation product was reduced relative to that of the *COX2* mRNA product. A similar though less-pronounced reduction in labeling after 10 min was previously observed (21). The strains used in that study were apparently triploid, based on the extremely low viability of meiotic spores they produced (unpublished results), which could account for relative differences in gene expression (6).

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Nuclear genotype	Mitochondrial genotype	Source
AFC6[a]	*MAT***a** *ura3-52 leu2-3,112 his3*Δ *arg8*::*hisG lys2* pAM2	*rho*$^+$ *cox2*::*ARG8*^{m} *var1*::*COX2*	This study
AFC15[a]	*MAT***a** *ura3-52 leu2-3,112 his3*Δ *arg8*::*hisG lys2 pet111*::*LEU2* pAM2	*rho*$^+$ *cox2*::*ARG8*^{m} *var1*::*COX2*	This study
SCS193[a]	*MAT***a** *ura3-52 leu2-3,112 his3*Δ *arg8*::*hisG lys2 imp1*::*kanMX4*	*rho*$^+$	Scott Saracco
AFC12[b]	*MAT***a**/α *ura3-52*/− *ade2-101*/+ *leu2-3,112*/+ *his3*Δ/+ *arg8*::*hisG*/*arg8*::*hisG lys2*/+ *kar1-1*/+ pAM2	*rho*$^+$ *var1*::*ARG8*^{m}	This study
AFC13[b]	*MAT***a**/α *ura3-52*/− *-ade2-101*/+ *leu2-3,112*/+ *his3*Δ/+ *arg8*::*hisG*/*arg8*::*hisG lys2*/+ *kar1-1*/+ pAM2	*rho*$^+$ *cox2*::*ARG8*^{m} *var1*::*COX2*	This study
AFC19[b]	*MAT***a**/α *ura3-52*/− *ade2-101*/+ *leu2-3,112*/+ *his3*Δ/+ *arg8*::*hisG*/*arg8*::*hisG lys2*/+ *kar1-1*/+ *imp1*::*kanMX4*/*imp1*::*kanMX4* pAM2	*rho*$^+$ *cox2*::*ARG8*^{m} *var1*::*COX2*	This study

[a] Congenic to D273-10B.
[b] From mating of strains congenic to D273-10B and strains congenic to DBY947.

Labeling of cells carrying the same nuclear and mitochondrial genomes, but homozygous for an *imp1*::*kanMX4* deletion, revealed accumulation of unprocessed pre-Cox2p (Fig. 1, lane 3). The electrophoretic mobility of this pre-Cox2p was identical to that produced by a haploid *imp1*::*kanMX4* mutant containing wild-type mitochondrial DNA (Fig. 1, lane 4). Similar results were obtained when the labeling pulse was shortened to 5 min, although overall incorporation of label was lower (unpublished data).

Thus, processing of newly synthesized pre-Cox2p generated by translation of the chimeric *var1*::*COX2* mRNA is dependent upon Imp1p, whose activity is located in the intermembrane space (18, 22). These results strongly suggest that, despite the presence of *VAR1* 5′- and 3′-UTRs on the chimeric mRNA which mislocalize pre-Cox2p synthesis (21), the N terminus of newly synthesized pre-Cox2p is readily translocated through the inner membrane. Apparently, the export apparatus in the inner membrane that translocates the N terminus (11) has ready access to the *var1*::*COX2* mRNA translation product. Based on the observation that other *Saccharomyces cerevisiae* mitochondrial 5′-UTLs are involved in localizing translation, we propose that the wild-type *VAR1* mRNA may also be translated at the surface of the inner membrane. A similar proposal has been made previously based on kinetic studies of mitochondrial protein synthesis (14).

However, our data are also consistent with the possibility that pre-Cox2p could be synthesized in a soluble milieu but rapidly and efficiently translocated through the inner membrane.

Translation of the wild-type *COX2* mRNA is activated through its 5′-UTL by the nuclearly coded membrane protein Pet111p (7, 16). However, there are also sites regulating translation of *COX2* within the coding sequence (2), as well as an amino acid sequence similarity between Cox2p and Pet111p that could underlie interactions involving these proteins (25). We therefore asked whether we could detect any effect of a *pet111* deletion mutation on expression of the *var1*::*COX2* chimeric gene. While *pet111* deletion prevented phenotypic expression of the *cox2*::*ARG8*^{m} gene in the control strain as expected, it did not further reduce the slow respiratory growth rate of the *var1*::*COX2* strain (Fig. 2) or synthesis of Cox2p

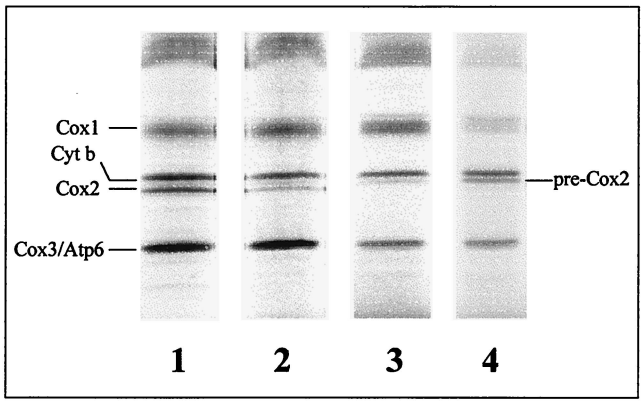

FIG. 1. Imp1p is required for processing of pre-Cox2p translated from the *var1*::*COX2* mRNA. Mitochondrial proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after radioactive pulse-labeling of the indicated strains (Table 1) for 10 min and detected by autoradiography. Lane 1, AFC12 [*COX2 var1*::*ARG8*^{m}]; lane 2, AFC13 [*cox2*::*ARG8*^{m} *var1*::*COX2*]; lane 3, AFC19 *imp1*::*kanMX4* [*cox2*::*ARG8*^{m} *var1*::*COX2*]; lane 4, SCS193 *imp1*::*kanMX4* [*COX2*].

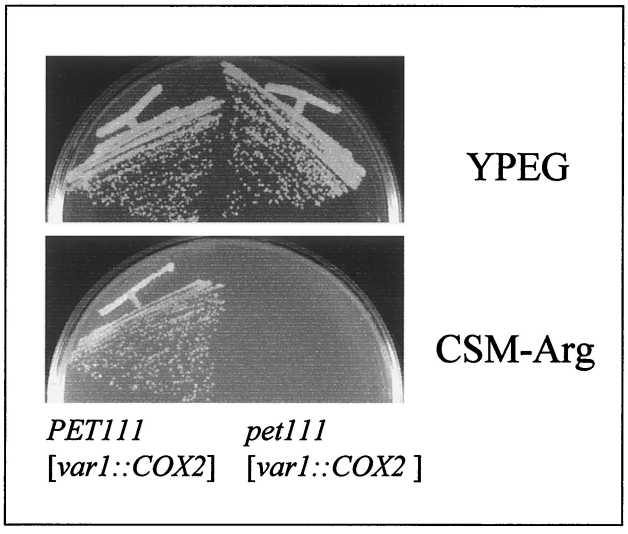

FIG. 2. Growth of *var1*::*COX2* strains on respiratory carbon sources is independent of Pet111p. The *var1*::*COX2* strain AFC6, left, and an isogenic *pet111* null mutant AFC15, right, were streaked on complete synthetic medium lacking arginine and on complete medium containing ethanol and glycerol as carbon sources (YPEG) and incubated at 30°C for 3 and 5 days, respectively.

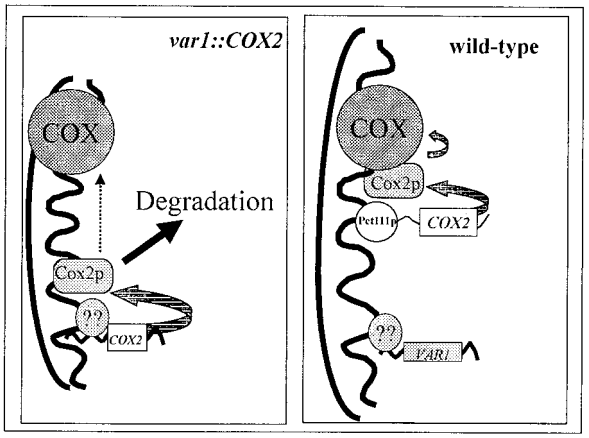

FIG. 3. Model for the translation of the *var1*::*COX2* chimeric mRNA and wild-type *COX2* and *VAR1* mRNAs in yeast mitochondria. We propose that the *VAR1* mRNA 5′-UTL directs synthesis of Cox2p on the matrix side of the inner membrane through the action of an unknown *VAR1* translational activator. Reduced incorporation of Cox2p into cytochrome *c* oxidase leads to rapid degradation of most of the newly synthesized protein.

from the chimeric mRNA in pulse-labeling experiments (unpublished results).

In conclusion, our evidence taken together with previous studies argues that the UTRs of the *VAR1* mRNA direct translation of coding sequence information to sites at or in close proximity to the membrane. We speculate that membrane-associated mitochondrial synthesis of Var1p could help nucleate the ribosome assembly process, which must also involve proteins imported through the inner membrane from the cytoplasm. However, these ribosome assembly sites would be distinct from locations where Cox2p can be efficiently assembled into the cytochrome *c* oxidase holoenzyme. The diagram in Fig. 3 summarizes this proposal, which suggests that Var1p is also likely to be translated at the surface of the inner membrane via the action of an as-yet-unidentified membrane-bound translational activator, while translation of the *COX2* mRNA is localized elsewhere on the membrane by its interaction with Pet111p. Thus, we propose the existence of distinct sites of assembly for mitochondrial ribosomes and cytochrome *c* oxidase.

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